HYBRIDIZATION OF LABORATORY STRAINS OF SIBLING SPECIES A AND B OF *ANOPHELES QUADRIMACULATUS*

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ABSTRACT. Adult mosquitoes of the *Anopheles quadrimaculatus* complex were collected from Montgomery County, AL (MON) and Alachua County, FL, (KBG) and laboratory stocks of species A and B were established through a selection procedure employing isofemale lines. Progeny from a cross of species B females to ORL males were usually semisterile females and sterile males. Progeny of the reciprocal cross were also semisterile females and sterile males, but the sex ratio was variable and ranged from normal to no males because of male mortality during the pupal stage. Conspecific crosses between strains from the two locations resulted in fertile offspring. Crosses between the sibling species from the two locations invariably gave semisterile females and sterile males (or lethal effects). This evidence confirmed previous data from hybridization and electrophoretic analyses of field populations indicating that *Anopheles quadrimaculatus* is a species complex.

INTRODUCTION

The nearctic *Maculipennis* complex of anopheline mosquitoes is comprised of several closely related species that are morphologically distinguishable (Kitzmiller et al. 1967). On the basis of the results of hybrid crosses and cytogenetic analysis of polytene chromosomes, *Anopheles freeborni* Aitken, *Anopheles aztecus* Hoffman, *Anopheles occidentalis* Dyar and *Anopheles earlei* Vargas were included as primary members of the nearctic complex. Hybrid crosses confirmed separate species status for these members, however, extensive homologies exhibited in the salivary gland polytene chromosomes signified close phylogenetic relationships. *Anopheles quadrimaculatus* Say and *Anopheles punctipennis* (Say) were considered borderline members of the complex.

Anopheles quadrimaculatus is the most important North American anopheline in the populous eastern United States, where it is a general nuisance, particularly in rice growing areas and reservoir systems, e.g., the Tennessee Valley Authority (TVA). Formerly, this species was an important vector of malaria. Recently, we have determined that *An. quadrimaculatus* is a complex composed of three sympatric, sibling species; provisionally named species A, B and C, species A and B are more closely related to each other than either is to C. The evidence accumulated thus far includes: (1) hybrid sterility in crosses between standard laboratory strains and field populations (collected in Florida, Alabama, Mississippi, Arkansas and Louisiana) (Lanzaro 1986)[3], (2) diagnostic banding patterns and fixed inversions on the polytene chromosomes of field populations (Kaiser and Seawright 1987, Kaiser et al. 1988), and (3) diagnostic enzyme loci (Lanzaro 1986[3], Narang et al. 1988).

Results of the initial hybridization experiments involving crosses of a standard species A laboratory stock to field-collected mosquitoes indicated that there were two sibling forms. Verification of the taxonomic status of species A and species B required crosses between known types. The work described in this paper summarizes our efforts in establishing strains of species A and species B from Alachua County, Florida and Montgomery County, Alabama, results of hybrid crosses verifying the status of each strain, and the frequencies of the sibling forms during the primary breeding season at the Montgomery collection site.

METHODS AND MATERIALS

On the basis of previous observations (Lanzaro 1986[3]), samples of field populations known to contain both species A and B were collected at two locations, 16 km east of Montgomery, Ala., (MON) and 3.2 km west of Gainesville, Fla. in Kanapaha Botanical Garden (KBG). The primary breeding areas for MON and KBG were a borrow pit and shallow lake, respectively, and both contained large amounts of aquatic vegetation. At each locale a deciduous forest provided tree holes that were the main daytime resting sites for adult mosquitoes. Adults were collected from tree holes with battery-powered aspirators, transferred to cages, and returned to the laboratory in chilled ice chests. In the laboratory adults were held at 26°C, ca. 80% RH, and maintained on a 10%

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sugar water solution. Females were blood-fed either on guinea pigs or humans. Induced copulation, using a modification of the technique of Baker et al. (1962), was necessary for all of the matings of field-collected mosquitoes. Gravid females were routinely traumatized by tearing one wing from the thorax to stimulate oviposition in individual vials. Culturing of the immature stages was done according to established procedures (Benedict and Seawright 1987).

In order to establish strains of species A and B, isofemale lines were established from the MON and KBG sites. Adult F_1 males from each line were mated to ORL (species A) females and also to siblings. The testes were removed from five-day old adult F_1 hybrid males from each family of the cross to ORL and examined under phase-contrast (187.5X). Testes were scored for size and the gross appearance of spermatids and sperm. For families without males, F_1 females were dissected and the size and general appearance of the ovaries were recorded. Isofemale lines that were compatible and incompatible with ORL were selected for further study.

Once established, the species A and B strains from MON and KBG were mated to each other and to ORL. The fertility of parental females was based on hatch of embryonated eggs and was averaged for the number of families for each cross; survival data represented the percentage of 1st instar larvae (based on hatch) that reached adulthood. The sex ratio of F1 progeny was also recorded. Five 5-day old males from each F_1 family were dissected and their testes were observed to assess hybrid sterility.

Males from 4 collections from the MON site were mated to females from the standard laboratory strain, ORL, to determine whether the frequencies of the sibling species varied during the primary breeding season. Testes from the adult F_1 hybrid males were scored as previously described.

RESULTS

Gravid MON and KBG females were collected and taken to the laboratory for the purpose of establishing isofemale lines that were genetically compatible or incompatible with ORL. Initially, 146 MON females were isolated and 16 laid egg batches. The remaining females were traumatized (wing removal) which resulted in 60 additional batches. Ninety-five percent of the egg batches hatched, which indicated that wing removal does not usually induce virgin females to deposit eggs. Sixty MON families were reared but only 25 were inbred and outcrossed to ORL because of the tedious nature of the forced copulation technique. Immature mortality reduced our sample to 13 families, of which 1 was compatible and 12 were incompatible with ORL (Table 1). Nine of the 12 incompatible lines contained both males and females and 3 contained only females. Female collections from KBG were handled in the same manner as that described for MON. Fifty-five lines were reared; 30 were inbred and outcrossed to ORL, and 21 produced F_1 hybrid data. Fifteen lines were compatible with ORL, and 6 were incompatible. Of the incompatible lines, 33% produced no males in crosses to ORL.

Three strains were selected for each location (Table 1). MON-A and KBG-A were compatible with the ORL species A strain. The lines that produced aberrant progeny were divided into groups with all-female offspring (designated MON-B_1 and KBG-B_1) and with female and male progeny (designated MON-B_2 and KBG-B_2). These incompatible lines (MON-B_1 and B_2 and KBG-B_1 and B_2) were eventually combined to form MON-B and KBG-B to facilitate maintenance. MON-B and KBG-B had a 1:1 sex ratio when inbred, and F_1 males and females appeared normal.

Table 1. Summary of data from establishing new laboratory strains of *Anopheles quadrimaculatus* from compatible (sp. A) and incompatible (sp. B) isofemale lines from Montgomery Co. (MON), AL and Kanapaha Botanical Gardens (KBG), Gainesville, FL.

Strain designation	No. of lines[a]	No. of matings[b]	F1 sex distribution[c] ♀♀	% ♀	♂♂	% ♂	Testes[c]
MON-A	1	25	224	48.6	237	51.4	Normal
MON-B_1	3	81	267	100	0	0	—
MON-B_2	9	208	834	73.4	303	26.6	Aberrant
KBG-A	15	227	1006	51.7	939	48.3	Normal
KBG-B_1	2	31	176	100	0	0	—
KBG-B_2	4	49	174	74.0	61	26.0	Aberrant

[a] Isofemale lines used to establish each strain.
[b] Matings used to establish each strain.
[c] Results of crosses to ORL.

Under laboratory conditions, all of the larvae of species B families had a green-body color. Although body color is usually rejected as a taxonomic character, in the case of these two sibling species, it was the only reliable way for us to separate the two forms. The species A isofemale lines were never all green. They contained an occasional green larva, but never in a ratio to nongreen that would indicate Mendelian inheritance. This character cannot be used for field-collected larvae, because of the environmental effects on the highly variable color patterns of larvae in natural populations, but it is useful in a laboratory environment.

Three species A lines (ORL-A, MON-A and KBG-A) and six species B lines (MON-B, MON-B_1, MON-B_2, KBG-B, KBG-B_1 and KBG-B_2) were maintained in our laboratory and used in crosses. Cross data are presented in Table 2. Species A lines produced fertile progeny when mated to each other, as did B lines; this indicated that A lines from MON and KBG were conspecific, as were the B lines from the two sites. Conversely, when A ♀ × B ♂ (AB) or the reciprocal cross was made, sterile males and semi-sterile females were produced. The mean fertility (%) of F_1 hybrid females was as follows: AB♀ × A♂ = 13.2 ± 11.2; AB♀ × B♂ = 10.0 BA♀ × A♂ = 17.1 ± 14.4; BA♀ × B♂ = 12.2 ± 9.7. When MON-B_1 males were used in crosses to A females, no F_1 males survived to the adult stage, but the F_1 progeny of the ORL ♀ × MON-B_2 cross always approximated a 3:2 sex ratio. When MON-B_1 and MON-B_2 were combined to make the MON-B strain, males from the new strain mated to ORL produced a higher percentage of F_1 males than ORL ♀ × MON-B_2.

Testes of sterile F_1 hybrid males were atrophied or absent. Atrophied testes typically contained a small number of spermatids with enlarged heads and shortened tails, but mature sperm were never present. In testcrosses, these males failed to produce progeny. Ovaries of F_1 hybrid females were approximately 25% the size of those of ORL and MON-B. This appeared to be due to incomplete development of the ovary resulting in fewer ovarioles (fecundity for A♀ × B♂ and B♀ × A♂ F_1 females averaged 119.5 and 122.5, respectively).

The results of the matings of MON males to ORL females, from the four separate collections, are shown in Table 3. The majority of the males were mated to more than one female; however, only 68.2% produced progeny. Most of the matings were incompatible (range =

Table 2. Summary of data from inbreeding and out-crossing strains of *Anopheles quadrimaculatus* species A and species B.

Cross (♀ × ♂)	No. of matings	Percent fertility (±SD)	No. of families	Percent survival (±SD)	F1 sex ratio		F1	
					% ♀♀	% ♂♂	Ova[a]	Tes[b]
ORL-A × ORL-A	42	87.3 ± 11.9	13	79.4 ± 19.0	50.5	49.5	N[c]	N
MON-A × MON-A	146	82.5 ± 17.7	30	61.2 ± 8.9	43.7	56.3	N	N
MON-B × MON-B	64	87.1 ± 14.7	24	68.1 ± 19.5	47.0	53.0	N	N
MON-B_1 × MON-B_1	145	79.4 ± 15.6	44	77.1 ± 16.2	47.7	52.3	N	N
MON-B_2 × MON-B_2	53	78.6 ± 16.5	12	69.9 ± 5.3	50.2	49.8	N	N
KBG-A × KBG-A	50	90.7 ± 12.6	14	64.6 ± 15.4	54.1	45.9	N	N
KBG-B × KBG-B	80	81.9 ± 15.5	22	62.4 ± 20.4	44.8	55.2	N	N
MON-A × ORL-A	42	83.8 ± 5.8	9	73.7 ± 14.1	48.8	51.2	N	N
ORL-A × MON-A	40	89.2 ± 3.4	8	72.6 ± 15.1	47.5	52.5	N	N
MON-B × ORL-A	24	76.4 ± 23.3	6	67.6 ± 17.4	51.3	48.7	A[d]	A
ORL-A × MON-B	29	75.4 ± 26.6	6	73.4 ± 24.5	52.8	47.2	A	A
MON-B_1 × ORL-A	32	78.3 ± 7.9	8	69.0 ± 11.5	49.8	50.2	A	A
ORL-A × MON-B_1	30	92.7 ± 1.2	6	37.8 ± 5.3	100	—	A	—
MON-B_1 × MON-A	59	86.4 ± 15.1	18	39.1 ± 14.6	51.6	48.4	A	A
MON-A × MON-B_1	55	81.3 ± 20.3	16	25.2 ± 6.9	100	—	A	—
MON-B_2 × ORL-A	14	77.4 ± 6.0	4	76.9 ± 6.2	50.4	49.6	A	A
ORL-A × MON-B_2	16	78.5 ± 6.4	4	80.4 ± 17.3	58.9	41.1	A	A
KBG-A × ORL-A	20	83.3 ± 6.8	6	64.4 ± 15.2	51.1	48.9	N	N
ORL-A × KBG-A	18	85.8 ± 3.4	7	71.0 ± 14.4	49.8	50.2	N	N
KBG-A × KBG-B	40	81.1 ± 12.6	9	49.4 ± 16.6	59.2	40.8	A	A
KBG-B × KBG-A	30	87.0 ± 12.1	10	64.7 ± 13.9	45.2	54.8	A	A
KBG-B × MON-B	29	79.1 ± 18.1	9	82.6 ± 8.9	47.7	52.3	N	N
MON-B × KBG-B	38	68.0 ± 19.9	9	77.8 ± 23.8	51.7	48.3	N	N

[a] ovaries,
[b] testes,
[c] normal and
[d] aberrant.

Table 3. Results of mating *Anopheles quadrimaculatus* males from four collections made during 1984 in Montgomery Co., AL, to females of the Orlando strain.

Collection date	No. males mated	No. of matings[a]	Compatible matings[b]		Incompatible matings[c]				Total no. males assayed
					No males		Males		
			No.	%	No.	%	No.	%	
June 17	70	161	3	6.7	13	28.9	29	64.4	45
July 26	71	176	10	17.5	16	28.1	31	54.4	57
August 29	75	197	7	12.7	18	32.7	30	54.6	55
October 19	75	134	8	19.5	6	14.6	27	65.9	41

[a] Males were mated to more than one female.
[b] Characterized by F_1 progeny that were reproductively normal.
[c] Characterized by F_1 progeny that were reproductively aberrant.

80.5–93.3%). Some families consisted of only females, and their ovaries were small and poorly developed. High pupal mortality was invariably associated with a lack of adult males, and microscopic examination revealed that most of the dead pupae were males. In contrast, the compatible matings produced F_1 progeny with a sex ratio of 1:1 and males with normal appearing testes that contained large numbers of mature sperm. The incompatible matings that produced males varied in sex ratio from 9:1 (female:male) to 1:1. The largest number of incompatible matings, 93.3%, was observed in the initial collection (June 17, 1984), and the fewest incompatible matings, 80.5%, were seen in the final collection (October 10, 1984).

DISCUSSION

These results confirm that the MON and KBG populations are both comprised of sympatric sibling species of *An. quadrimaculatus*. The ORL strain, which was colonized about 40 years ago, and the A-lines from MON and KBG, are all species A. The B-lines, all of which produced sterile males in crosses to species A, are all lines of sibling species B. Although the results of crossing species A females to species B males varied, because some crosses produced F_1 males and some did not, when these different B-lines were bred to each other all of the F_1 families generated fertile males and females. The "factor(s)" in the B-lines that were responsible for all-female families remained in that line as long as it was inbred. However, when crossed to another B-line that produced hybrid males, the resulting B-line always propagated sterile F_1 males when crossed to species A females. At present, the factors responsible for the hybrid male survival polymorphism are not known.

The initial discovery of sibling species of *An. quadrimaculatus* was quite accidental, in that a family of species B was selected from isofemale lines from Stuttgart, AR because all of the larvae in the family had a green-body phenotype. Males of this family were crossed to ORL, and no F_1 males were produced. The frequency of species B was quite low at Stuttgart, and a subsequent survey (Lanzaro 1986[3]) indicated that species B was more numerous elsewhere, e.g., at MON and KBG, thus facilitating the establishment of wild lines of species A and B in our laboratory.

The most common larval pigmentation in field collections of species A varies from tan (usually males) to brownish purple (usually females). Larvae with green pigmentation are also fairly common, especially when the algal content of breeding water is high, but this form of green pigmentation is environmental rather than genetic. In species B, the fourth instar male larvae are always bright green and the females contain pigmentation that ranges from dark green to greenish-purple. Kaiser et al. (1988) collected species B from 6 locations in 4 southeastern states and the green larval pigmentation trait was consistent throughout those populations.

The differences in hybrid survival that we noted when we crossed MON-B_1 and MON-B_2 males to ORL are not without precedence. Almost every possible situation has been recorded for crosses of sibling species of anophelines. For example, these include, unidirectional male sterility (Miles 1981, Coetzee 1983), bidirectional male sterility (Davidson 1964, Davidson and Hunt 1973), all-male progeny (Davidson 1964) and sex ratio distortion in favor of females (Mahon and Meithke 1982).

Kaiser et al. (1988) used fixed inversion differences to identify the sibling forms of *An. quadrimaculatus* in 8 widely distributed locations, and reported finding species A at all of the sites and species B at 5 sites. A predominance of species B was found only at the Montgomery location. More recently, ten additional populations have been observed (unpublished data) and species A and species B were found at 10 and 6 of the locations, respectively. Three of

the six populations (in Mississippi, Tennessee, and Florida) were predominantly species B.

It is important to determine the range of the sibling species of *An. quadrimaculatus* s.l., and to this end a taxonomic key would be useful. The sibling species can be distinguished cytologically (Kaiser et al. 1988) and electrophoretically (Lanzaro 1986[3]), although both techniques are tedious for large samples. Carlson and Service (1979) analyzed cuticular hydrocarbons to differentiate members of the *An. gambiae* complex, and this method can also be used to separate the *An. quadrimaculatus* s.l. complex (personal communication, D. Carlson); however, it is not suitable for the rapid analysis of large sample sizes.

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